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Research Article

Exploration of Amylases Producing Competency of *Helicoverpa armigera* Gut Bacterial Strain, *Bacillus subtilis* RTSBA6 6.00

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ABSTRACT

The *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) is a polyphagous insect pest of agriculturally important crops. The alkaline gut of this insect pest possesses diverse bacterial communities which may assist in digestive physiology. As part our investigations of understanding the role of gut bacterial communities in insect gut, here amylase producing competency of earlier identified *H. armigera* gut bacterial strain, i.e., *Bacillus subtilis* RTSBA6 6.00 is reported. Initial screening for amylase activity was assessed by starch agar plate. Upon 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis amylase zymography, bacterial culture supernatant produced seven amylase bands on the gel. The observed molecular weights of amylases were 191.2 KDa, 158.0 KDa, 131.7 KDa, 54.0 KDa, 31.3 KDa, 67.2 KDa, and 44.6 KDa, respectively. Considerable amylase activity was observed in neutral to alkaline pH with optimum at pH 6.8. The optimal activity temperature of amylases was found to be 50°C, and the activity decreased dramatically at temperatures above 75°C.

Keywords: Amylase, *Bacillus subtilis* RTSBA6 6.00, *Helicoverpa armigera,* zymography

INTRODUCTION

The symbiotic associations of gut bacterial communities with the host insects are imperative for the host fitness leading to nutrition, development, resistance to pathogens, and reproduction.[1,2] This class of microorganisms is not only contributing in metabolic activities but also behavior of insect hosts.[3,4] Improved digestion efficiency of host insects is attributed to digestive enzymes expressed by gut bacterial communities.[3] The secretion of cellulases and lignases enzymes by gut bacterial communities provides glucose and fatty acid to host insect.[5] The bacterial communities associated with lepidopteran insects recently gained attention from scientific community. Some of these insects are considered the most damaging agricultural and forest pests worldwide. *Helicoverpa armigera* is a devastating insect pest of agricultural crops such as cotton, chickpea, pigeon pea, tomato, sunflower, okra, and corn.[6] The use of modern chemical and biological insecticides has limited success in management due to adaptation acquired by this insect pest.[7,8]

The detailed exploration of gut bacterial community and their contribution in host digestive physiology and adaptation to insecticides is necessary to cope of these insect pests. The multitrophic association of gut bacterial communities with *H. armigera* explored.[9-11] The bacterial strain *Bacillus subtilis* RTSBA6 6.00 isolated from gut of *H. armigera* is reported to secrete proteases enzymes.[10] This proteolytic arsenal may help *H. armigera* to contribute in digestion and adaptation. Earlier, gut bacterial strain with serine protease activity from velvetbean caterpillars was reported.[12]

In present communication, the *H. armigera* gut isolated bacterial strain *B. subtilis* RTSBA6 6.00 explored further for its ability to produce amylase enzymes. Amylases are digestive enzymes that act in the first step of maltopolysaccharide digestion. They cleave α 1–4 glycosidic linkage exists in starch. Insects feed on plants for carbohydrates (starch and cellulose) during different stages of their lives and dependent on starch hydrolyzing enzymes for its survival. The amylase action is necessary to improve digestive performance of insects leading to survival within different living conditions and increase their biological fitness.[13] Furthermore, the bacterial communities expressing amylases is receiving more significance due to their potential role in food, pharmaceutical, and fine chemical

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industries. We identified and characterized several isoform of amylases produced by *B. subtilis* RTSBA6 6.00. Initial detection of amylase activity was carried on starch agar plate. The optimum temperature and pH were estimated by solution assays. Amylase isoforms analysis was done by zymography technique.

MATERIALS AND METHODS

Isolation, Cultivation, and Identification of Bacteria

Earlier isolated *H. armigera* gut bacterial strain, identified, and characterized by fatty acid methyl ester-based analysis[10,14] were used in the present investigation.

Detection of Amylase by Agar-plate Method

Primary screening of bacterial strain *B. subtilis* RTSBA6 6.00 for amylase activity was done by starch hydrolysis on the solid media containing 1% starch, 0.3% beef extract, and 1.2% agar. Strain was inoculated on the media plate and incubated for 24 h at 37°C.[15] Plate was stained with starch specific Lugol's solution (0.1% iodine and 1% KI), and zone of clearance (α-amylase activity) was observed visually.

Amylase Production

The strain of *B. subtilis* RTSBA6 6.00 was cultured in starch broth. The starch broth was prepared by mixing all ingredients except starch together in Milli-Q water. The pH of broth was adjusted to alkaline condition before sterilization at 121°C for 15 min. Starch solution was separately sterilized at 121°C for 15 min. These solutions were mixed aseptically before use. The culture was incubated at room temperature on shaker for 48 h. The cells were harvested by centrifugation at 6000 rpm, 4°C for 20 min. The supernatant was used as crude enzyme source for further studies. The protein concentration was estimated by Folin–phenol reagent[16] using bovine serum albumin as standard.

Starch Zymography

For starch zymography, 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli.[17] 0.1% soluble starch was incorporated in the gel. About 100 μg of crude protein was loaded on gel with standard molecular weight markers (Genei). After electrophoresis, gel was washed twice at room temperature in a solution of 2.5% Triton-X 100 for 30 min. After gentle washing with Milli-Q water, the gel was incubated in 0.1 M Tris–HCL (pH 8) at 37°C for overnight. The gel was stained with Lugol's solution, and the zones of starch clearance were detected.

Molecular Weight Determination

The molecular weight of α-amylase isoforms was determined by comparing its electrophoretic mobility with that of standard proteins having known molecular weights. SDS-PAGE was carried out using 7% resolving gel.[17]

Enzyme Activity Assay

Amylase activity was determined by spectrophotometric method as described by Fisher and Stein.[18] According to the procedure, 1.0 ml of crude enzyme into a test tube and 1 ml of 1% soluble starch in 0.1 M Tris–HCL (pH 8) were added in test tube. The test tubes were covered and incubate at 35°C for 15 min in water bath. Then, 2.0 ml DNSA (1% 3, 5-dinitrosalicylic acid, 30% sodium potassium tartrate, and 0.2 M NaOH) reagent was added in each tube to stop the reaction and kept in boiling water bath for 5 min. After cooling at room temperature, the absorbance was read at 540 nm by spectrophotometer.

Effect of pH on Amylase Activity

The effect of different pH on the activity of amylase was examined using standard DNSA assay as described previously. Amylolytic pattern of amylase at different pH was observed on 7% starch zymography. After electrophoresis, the gel was cut and the stripes of gel were washed twice at room temperature in a solution of 2.5% Triton-X 100 for 30 min. Each strip of gel was incubated in 0.1 M acetate buffer for pH 2.8, 3.8; 0.1 M phosphate buffer for pH 5.8 and 6.8;0.1 M Tris–HCL pH 7.8, 8.8 and 9.8. 0.1 M Tris–NaOH for pH 11.8, respectively at 37°C for overnight and the gel was stained with Lugol's solution and the zones of starch clearance were detected.

Effect of Temperature on Enzyme Activity

The influence of heating on the catalytic activity of amylases was determined by pre-incubation for 10 min at different temperatures ranging from 35, 40, 45, 50, 55, 60, and 65°C, followed by cooling and measuring the remaining activity. The stability of amylase at different temperature was observed by loading pre-incubated sample on 10% starch zymography as described previously.

RESULTS AND DISCUSSION

This study deals with deciphering amylase producing competency of bacterial strain which was isolated and identified as *B. subtilis* RTSBA6 6.00 from gut of fourth instar larvae of *H. armigera*.[10] The strain was Gram-positive spore forming. This strain was screened for amylase activity using starch agar plate method. The starch agar is a differential medium that assesses the ability of an organism to produce starch hydrolyzing enzymes, namely amylase. In principle, the inoculated microorganisms hydrolyze starch present in medium upon incubation at an appropriate temperature. Starch can be stained with Lugol's solution which turns blue-black. Absence of the blue-black color indicates starch hydrolysis. Here upon initial detection, the presence of clear zone of starch hydrolysis was observed around the growth of *B. subtilis* RTSBA6 6.00 on the plate [Figure 1]. Amylase cleaves the starch into di- and oligosaccharides which are transported into the cell to be catabolized. In general, all *Bacillus* species have potential to produce the amylase.[19]

The pure culture of *B. subtilis* RTSBA6 6.00 was cultured in starch broth for amylase production with optimum parameters. The supernatant recovered after centrifugation was used for further characterizations. The amylase in

the supernatant was separated into seven bands on the non-reducing 7% SDS-PAGE amylase zymography [Figure 2]. This is an electrophoretic technique used to study amylase by means of thin gels containing copolymerized starch, under non-reducing conditions. The formation of clear zones against a dark background in the gel stained with Lugol's solution indicated the presence of amylolytic activity.[20] The zone of starch clearing corresponded to a migration distance of amylase by comparison to the molecular weight standards used in the study. These amylases showed differential mobility on the gel resulting different molecular masses. The major amylase bands were observed with molecular weights 191.2 KDa, 158.0 KDa, 131.7 KDa, 54.0 KDa, and 31.3 KDa, respectively. Two moderate intensity bands were also observed with molecular weights 67.2 KDa and 44.6 KDa.

In our study, we found that, amylase isolated from *B. subtilis* RTSBA6 6.00 showed optimum activity in slightly neutral pH (6.8) as shown in Figures 3 and 4. This parameter assessed under standard assay conditions and zymographically. These amylases had a relatively low activity in acidic pH range, i.e., pH 2.8. The amylases were found to be most active at the neutral to alkaline pH range.

The optimal pH for amylases of most of the insect species was found to be neutral to alkaline pH range, which is near to our finding for *B. subtilis* RTSBA6 6.00 isolated from gut of *H. armigera*.[21] The stability of these amylases in the alkaline region indicates that they may be potentially used in digestive physiology of insect.

Beside pH, temperature is crucial factors that affect enzymatic activity. Bacterial amylases were optimally active at temperatures ranging from 35 to 55°C, with a decline in activity above 75°C [Figures 5 and 6]. The optimum temperature was found to be 50°C. Beyond temperature 75°C decline in activity might be attributed to denaturation of amylases. Earlier amylase with optimum activity in slightly higher temperature

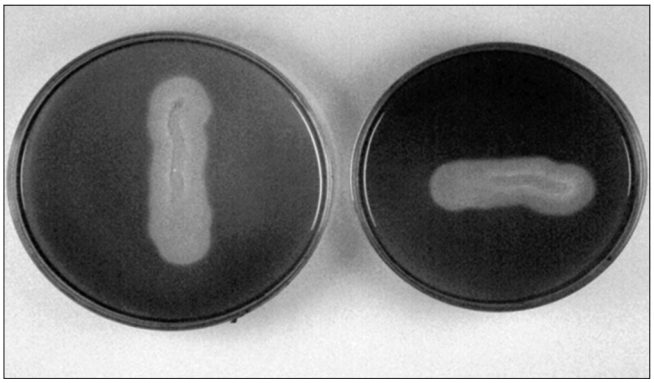

Figure 1: Screening of *Bacillus subtilis* RTSBA6 6.00 for amylase activity. Strain was streaked on agar plate containing 1% starch, incubated for 24 h at 37°C. The clear zone indicated the hydrolysis of starch

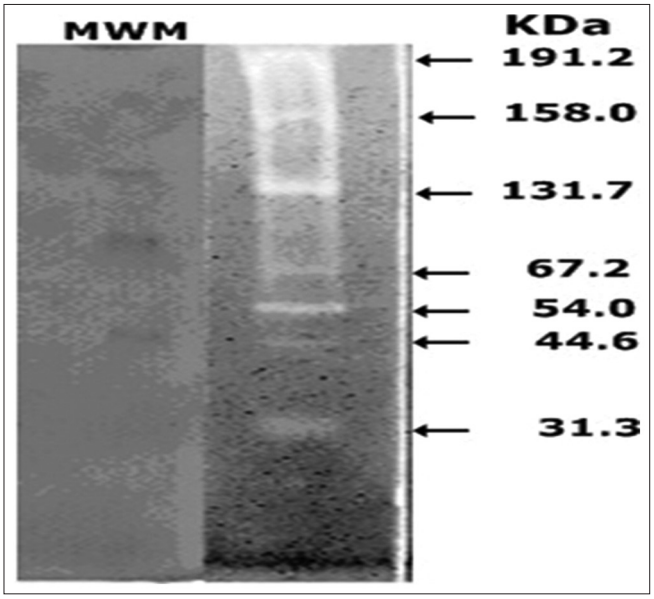

Figure 2: Non-reducing 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis amylase zymography of proteases produced by *Bacillus subtilis* RTSBA6 6.00

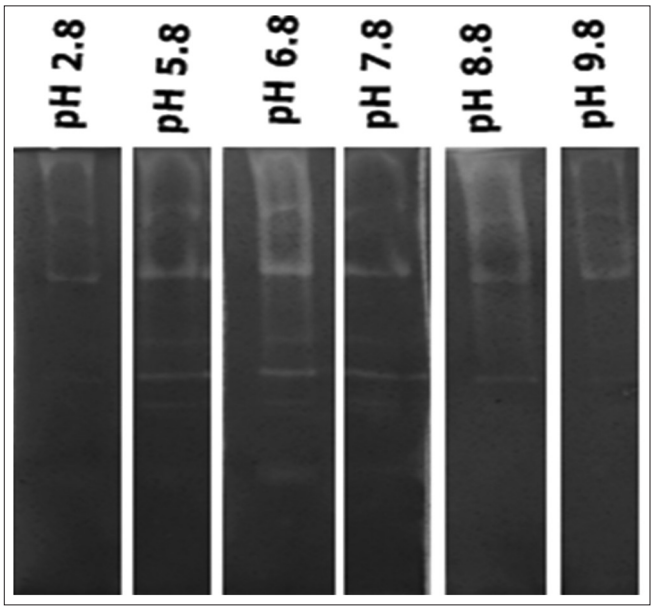

Figure 3: Non-denaturing 7% sodium dodecyl sulfate polyacrylamide gel electrophoresis zymography showing the influence of pH on the amylase activity of *Bacillus subtilis* RTSBA6 6.00

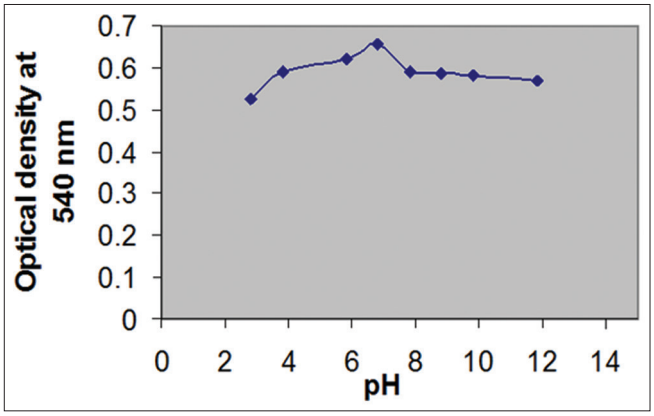

Figure 4: Effect of different pH values on amylolytic activity of *Bacillus subtilis* RTSBA6 6.00. Samples were assayed by standard procedure using starch as substrate

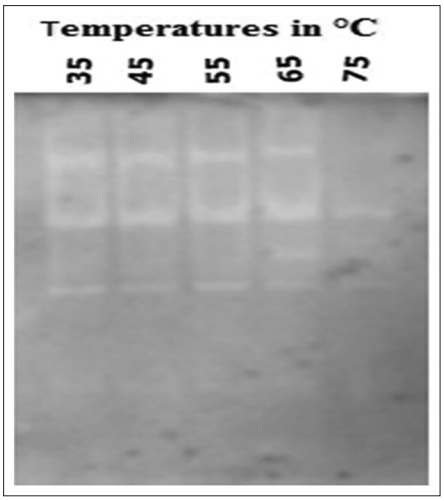

Figure 5: Non-denaturing 7% starch sodium dodecyl sulfate polyacrylamide gel electrophoresis showing the influence of temperature on the amylase activity of *Bacillus subtilis* RTSBA6 6.00

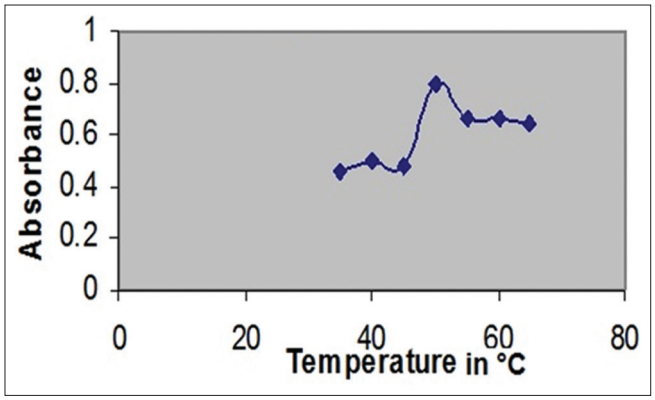

Figure 6: Effect of different temperatures on amylolytic activity of *Bacillus subtilis* RTSBA6 6.00. Samples were assayed by standard procedure using starch as substrate mentioned in material and methods

range than the physiological temperature was reported form *Bacillus* species.[22]

The gut of *H. armigera* has an alkaline pH, and *B. subtilis* RTSBA6 6.00 was found to grow and degrade starch at neutral to alkaline pH. The presence of bacteria producing several amylases with optimal activity in alkaline region could be advantageous to *H. armigera*. Here, it might be possible that these bacterial amylases contribute to amylolytic arsenal of *H. armigera*, which feeds on carbohydrate rich part of plants. Optimal activity at near alkaline pH in lepidopterans may be an adaptive response for the digestion of their diets.[23] The characterization of microbial amylase with thermo stability and activity in wide pH range may also found industrial applications, and hence, our future investigation will focus on the detailed investigations of these microbial amylases.

REFERENCES

1. N. A. Moran, P. H. Degnan, S. R. Santos, H. E. Dunbar and H. Ochman. "The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes." *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, pp. 16919-16926, 2005.
2. J. C. Clemente, L. K. Ursell, L. W. Parfrey and R. Knight. "The impact of the gut microbiota on human health: An integrative view". *Cell*, vol. 148, pp. 1258-1270, 2012.
3. R. Dillon and V. Dillon. "The gut bacteria of insects: Nonpathogenic interactions". *Annual Review of Entomology*, vol. 49, pp. 71-92, 2004.
4. F. M. Pilon, L. E. Visotto, R.N.C. Guedes and M. G. A. Oliveira. "Proteolytic activity of gut bacteria isolated from the velvet bean caterpillar *Anticarsia gemmatalis*". *Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology*, vol. 183, pp. 735-747, 2013.
5. J. A. Breznak and A. Brune. "Role of microorganisms in the digestion of lignocellulose by termites". *Annual Review of Entomology*, vol. 39, pp. 453-487, 1994.
6. V. D. Parde, H. C. Sharma and M. S. Kachole. "*In vivo* inhibitions of *Helicoverpa armigera* gut pro-proteinase activation by non-host plant protease inhibitors". *Journal of Insect Physiology*, vol. 56, no 9, pp. 1315-1324, 2010.
7. A. A. Shinde, F. K. Shaikh, P. P. Gadge, M. V. Padul, S. P. Govindwar and M. S. Kachole. "Conserved nature of *Helicoverpa armigera* gut bacterial flora on different host plants and in vitro interactions with PI proteins advocates role in host digestive physiology". *Journal of the Saudi Society of Agricultural Sciences*, vol. 18, pp. 141-149, 2017.
8. H. Sharma, M. Dhillon and R. Arora. "Effects of *Bacillus thuringiensis* dendotoxin-fed *Helicoverpa armigera* on the survival and development of the parasitoid *Campoletis chlorideae*". *Entomologia Experimentalis et Applicata*, vol. 126, pp. 1-8, 2008.
9. P. K. Mishra and S. M. Tandon. "Gut bacterial flora of *Helicoverpa armigera* (*Lepidoptera*, *Noctuidae*)". *Indian Journal of Microbiology*, vol. 43, no. 1, pp. 55-56, 2003.
10. A. A. Shinde, F. K. Shaikh, M. V. Padul and M. S. Kachole. "*Bacillus subtillis* RTSBA6 6.00, a new strain isolated from gut of *Helicoverpa armigera* (*Lepidoptera*: *Noctuidae*) produces chymotrypsin-like proteases". *Saudi Journal of Biological Sciences*, vol. 19, pp. 317-323, 2012.
11. P. N. Gayatri, A. Ojha, M. K. Kajla, A. Raj and R. Rajagopal. "Host plant induced variation in gut bacteria of *Helicoverpa armigera". PLoS One*, vol. 7, pp. e30768, 2012.
12. L. E. Visoˆtto, M. G. A. Oliveira, A. O. B. Ribon, T. R. Mares-Guia and R. N. C. Guedes. "Characterization and identification of proteolytic bacteria from the gut of the velvetbean caterpillar (*Lepidoptera*: *Noctuidae*)". *Environmental Entomology,* vol. 38, no. 4, pp. 1078-1085, 2009.
13. R. Kaur, N. Kaur and A. K. Gupta. "Structural features, substrate specificity, kinetic properties of insect α-amylase and specificity of plant α-amylase inhibitors". *Pesticide Biochemistry and Physiology*, vol. 116, pp. 83-93, 2014.
14. J. S. Buyer. "Improved fast gas chromatography for FAME analysis of bacteria". *Journal of Microbiological Methods*, vol. 54, pp. 117-120, 2003.
15. S. Sharmin, M. D. Towhid and M. N. Anwar. "Isolation and characterization of a protease producing bacteria *Bacillus amoviv-orus* and optimization of some factors of culture conditions for protease production". *Journal of Biological Sciences*, vol. 5, no. 3, pp. 358-362, 2005.
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall. "Protein measurement with the folin phenol reagent". *Journal of Biological Chemistry*, vol. 193, pp. 265, 1951.
17. U. K. Laemmli. "Cleavage of structural proteins during the assembly of the head of bacteriophage T4". *Nature*, vol. 227, pp. 680-685, 1970.
18. E. H. Fisher and E. A. Stein. "Alpha amylase from human saliva". *Biochemical Preparations*, vol. 8, pp. 27-32, 1961.
19. G. G. Priest. "Extracellular enzyme synthesis in the genus *Bacillus*".

Bacteriological Reviews, vol. 41, no. 3, pp. 711-753, 1977.
20. A. Andrades and L. M. Contreras. Amylase zymography". *Methods in Molecular Biology*, vol. 1626, pp. 301-308, 2017.
21. F. Zeng and A. C. Cohen."Partial characterization of a-amylase in the salivary glands of *Lygus hesperus* and *Lygus lineolaris*". *Comparative Biochemistry and Physiology Part B*, vol. 126, pp. 9-16, 2000.
22. P. C. Aryati, A. Pangastuti and S. L. A. Sari. "Amylase Production Potentials of Bacterial Isolates Obtained from the Gut of *Oryctes rhinoceros* Larvae". IOP Conference Series: Materials Science and Engineering, 2017.
23. T. M. Clark. "Evolution and adaptive significance of larval midgut alkalinization in the insect superorder Mecopterida". *Journal of Chemical Ecology*, vol 25, pp. 1945-1960, 1999.